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Forskolin regulation of liver membrane adenylyl cyclase

Lutz BIRNBAUMER, Dominique STENGEL, Martine DESMIER, and Jacques HANOUNE

Unité de Recherches, Institut National de la Santé et de la Recherche Médicale Unité-99, Hôpital Henri Mondor, Créteil

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The effects of forskolin on rat liver plasma membrane adenylyl cyclase were studied. The diterpene stimulated the V_{max} of the enzyme system with apparent K_m values of 3 – 5 μM. Stimulations were marked both in the absence (20-fold over control) as well as in the presence of various stimulators such as GTP, Guo*PP*[NH]*P*, NaF alone or in combination with glucagon. Except with GTP, where stimulations of activities by forskolin and the nucleotide were synergistic (more than additive), stimulations of combinations of Guo*PP*[NH]*P*, NaF or glucagon with forskolin were additive.

Forskolin did not alter significantly the apparent K_m values of the enzyme for MgATP or MnATP or the apparent K_a values (concentrations giving stimulations that are 50 % of maximum) for Mg or Mn ions, GTP, Guo*PP*[NH]*P* or NaF. Forskolin caused a decrease in the concentration of glucagon required for half-maximal stimulation from 5 μM to 1.5 μM. Except for this effect on the K_a for the glucagon, the only kinetic parameter altered was the V_{max} under all conditions tested.

Although proteolysis stimulated liver membrane adenylyl cyclase under control conditions, it did not enhance forskolin-stimulated activities. More extensive proteolysis, which resulted in decreased activities in the absence of forskolin, also resulted in reduced forskolin-stimulated activities.

'Uncoupling' of the guanine-nucleotide-binding regulatory component, that mediates guanine nucleotide stimulation by addition of 30 mM $MnCl_2$, did not result in 'uncoupling' of forskolin stimulation. The data indicate that the diterpene forskolin stimulates adenylyl cyclase activity by a novel mechanism that differs from that by which NaF or guanylyl nucleotides affect this membrane-bound system and that the diterpene should be a useful tool with which to explore as yet unrecognized modes of regulation of cyclic AMP production.

Forskolin is a hypotensitive and cardiostimulatory diterpene that enhances adenylyl cyclase activity in most intact cells and plasma membranes studied (for review, see [1]). The enhancement of activity elicited by this drug is remarkable. It is almost always more than that elicited in the same system by hormones, by non-hydrolyzable GTP analogues or by the non-specific stimulator NaF [2, 3]. The mechanism and site of action of forskolin are centers of research, for it may well be an important tool in investigations on the basic and hormonal regulation of adenylyl cyclases. Seamon and Daly showed that forskolin stimulates Mg-dependent adenylyl cyclase activity in the so-called regulatory-component-deficient membranes of *cyc*$^-$ S49 lymphoma cells and these authors suggested that the effect(s) of the drug occur independently of the presence or absence of the regulatory component of adenylyl cyclase. However, other data point to a possible action of forskolin on an regulatory component, distinct from the catalytic unit of the system [3 – 5]. This article reports studies that were carried out to characterize the properties of forskolin-simulated liver membrane adenylyl cyclase in terms of its actions to modify kinetic parameters such as V_{max} and apparent K_m values for ATP and Mg ions, as well as in terms of the effect it may have on the requirements of the system for guanine nucleotides, fluoride ions and hormones.

Abbreviation. Guo*PP*[NH]*P*, guanosine 5′-[β,γ-imino]triphosphate.

MATERIALS AND METHODS

Materials

Forskolin was purchased from Calbiochem or obtained as a gift from Hoechst (France). ATP (catalogue no. A 2383), creatine phosphokinase, phosphocreatine, and cyclic AMP were obtained from Sigma. [α-^{32}P]ATP was from Amersham International (Great Britain), and cyclic [8-^{3}H]AMP was from the Çommissariat à l'Energie Atomique (Saclay, France). All other reagents were from standard sources.

Methods

Rat liver plasma membranes were prepared according to Neville [6]. Adenylyl cyclase activity of rat liver plasma membranes was measured in the presence of 0.5 mM ATP, 1 mM EDTA, 5 mM $MgCl_2$ or 2 mM $MnCl_2$, 1 mM cyclic AMP, 26 mM phosphocreatine, 25 U/ml creatine phosphokinase, 50 mM Tris/HCl pH 7.6 in the presence of 15 – 20 μg enzyme protein. Incubations were performed at 30 °C for 10 min in a final volume of 60 μl, and the reactions were terminated by a modification [7] of the procedure of White [8].

Calculation of unbound divalent cation (M^{2+}) concentrations. Total divalent cations (MCl_2), required to give the

desired concentrations of M^{2+} during adenylyl cyclase assays, were calculated according to the equation:

$$[M]_{total} = [M^{2+}]\left(1 + \frac{[A]_{tot}}{[M^{2+}] + K_A} + \frac{[B]_{tot}}{[M^{2+}] + K_B} + \frac{[C]_{tot}}{[M^{2+}] + K_C}\right)$$

where $[A]_{tot}$, $[B]_{tot}$ and $[C]_{tot}$ represent the total concentrations of added ATP, EDTA and creatine phosphate; K_A, K_B and K_C are the equilibrium dissociation constant of $MATP^{2-}$, MEDTA and M creatine phosphate at pH 7.6; $[M]_{total}$ is the concentration of MCl_2 added and M^{2+} is the resulting concentration of unbound divalent cation. The equilibrium dissociation constants used in these calculations were 60 μM for $MgATP^{2-}$ and 17 μM for $MnATP^{2-}$ [9–12], 400 nM for MgEDTA and 0.017 nM for MnEDTA [13, 14] and 50 mM for Mg and Mn creatine phosphate [15].

RESULTS

Effects of forskolin on liver membrane adenylyl cyclase

Table 1 illustrates the effect of forskolin on various types of adenylyl cyclase activities as seen upon addition of guanine nucleotides and NaF, alone and in combination with glucagon. From these data it became clear that forskolin, although effective under all conditions tested, acts on this system in a complex manner. Thus, while on the one hand the combination of the diterpene and GTP appears superadditive (i.e. synergistic), the combination with Guo*PP*[NH]*P* in the absence or presence of glucagon leads to final activities that can be considered as the result of additive actions between the two types of stimuli, and the combination with NaF resulted in activities that were somewhat less than additive. This difference in the type of additivity observed was not due to the use of subsaturating concentrations of the stimulators, for as shown in Fig. 1, the concentrations of forskolin used in the experiment of Table 1 (100 μM) is saturating both in the absence and in the presence of either guanine nucleotide Guo*PP*[NH]*P* or NaF. In this and other experiments in which we explored the dose-response relationship between forskolin and stimulation of activity, the concentration of the drug giving stimulations that were 50% of the respective maxima were between 3 μM and 6 μM regardless of whether assayed in the absence or the presence of 10 μM Guo*PP*[NH]*P* or 10 mM NaF. Fig. 2 illustrates experiments in which the possible effects of forskolin on the potency of the actions of GTP, Guo*PP*[NH]*P* and glucagon were investigated. The results showed that the stimulator forskolin had only slight effects, although not significant, on the concentrations of GTP, Guo*PP*[NH]*P* or glucagon required for half-maximal stimulation of activity, eliciting decreases that varied in several experiments between 2.0–4.0-fold for Guo*PP*[NH]*P* and GTP, and 1.5–2.5-fold for glucagon.

Fig. 3 shows the effect of forskolin on the dose-response curves for NaF stimulation of liver adenylyl cyclase. Although forskolin did not alter significantly the concentration of NaF that elicits half-maximal stimulation at 5 mM $MgCl_2$, it appeared to alter significantly the sensitivity of the system to the inhibitory action of the halide. The appearance of the inhibitory effect of fluoride ion on fluoride-stimulated activity upon forskolin addition was related in some manner to the concentration of $MgCl_2$ used in the assay. Increasing the concentration of the divalent cation to 10 mM practically abolished the inhibitory effect of 20 mM NaF observed at 5 mM $MgCl_2$ (Fig. 3).

Table 1. *Effect of forskolin and glucagon on adenylyl cyclase activity in liver plasma membranes assayed in the absence or presence of GTP, Guo*PP*[NH]*P *and NaF*

Liver membranes (20 μg protein) were assayed for adenylyl cyclase activity as described under Materials and Methods. Incubations were for 10 min at 30 °C. Values are means ± SD of triplicate determinations and are recorded as nmol cAMP formed/mg protein in 10 min. When present, forskolin was 100 μM, glucagon was 0.1 μM, guanine nucleotides were 10 μM and NaF was 10 mM. Values in parentheses correspond to calculated additive cyclase activities

Additions to assay	Adenylyl cyclase activity	
	without forskolin	with forskolin
	nmol cAMP/mg protein	
None	0.32 ± 0.05	2.90 ± 0.10
GTP	0.88 ± 0.03	4.45 ± 0.10 (3.78)
Guo*PP*[NH]*P*	2.18 ± 0.05	6.90 ± 0.10 (5.08)
NaF	2.58 ± 0.10	5.00 ± 0.10 (5.48)
Glucagon	2.15 ± 0.15	5.34 ± 0.10 (5.05)
Glucagon + GTP	4.00 ± 0.10	8.26 ± 0.50 (6.90)
Glucagon + Guo*PP*[NH]*P*	5.39 ± 0.05	9.02 ± 0.60 (8.29)

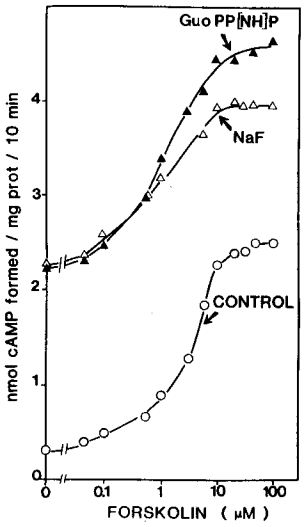

Fig. 1. *Effect of increasing concentration of forskolin in the absence or presence of effectors.* Adenylyl cyclase activity of rat liver plasma membranes was assayed in the presence of MgATP as described in Materials and Methods in the absence (○) or presence of 10 mM fluoride (△) and 10 μM Guo*PP*[NH]*P* (▲). Adenylyl cyclase activities are expressed in nmol cyclic AMP formed/mg protein in 10 min at 30 °C

Effects of forskolin on the kinetic parameters of adenylyl cyclase activity in liver plasma membranes

The above-described effect of forskolin to increase the dependence of the fluoride-stimulated activity on $MgCl_2$, led us to investigate which of the basic kinetic parameters of the liver adenylyl cyclase system is affected by forskolin. The parameters explored included not only the dependence on the divalent cation, but also on the substrate, MATP. As summarized for

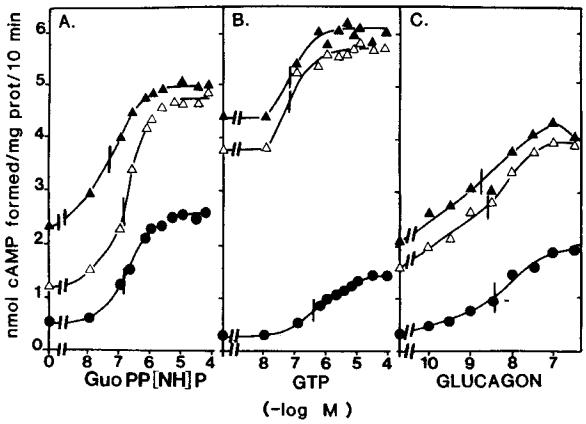

Fig. 2. *Effect of forskolin on stimulation of adenylyl cyclase by GuoPP[NH]P GTP or glucagon.* The assays were performed as described in Materials and Methods in the presence of varying concentrations of Guo*PP*[NH]*P* (A), GTP (B) or glucagon (C) without (●) or with 30 μM (△), and 100 μM (▲) forskolin in the assay medium. Activities are expressed in nmol cyclic AMP formed/mg protein in 10 min at 30 °C

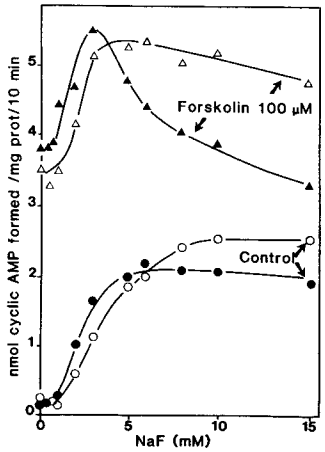

Fig. 3. *Effect of forskolin on stimulation of adenylyl cyclase by fluoride ions in the presence of two magnesium concentrations.* Rat liver plasma membrane adenylyl cyclase was assayed in the presence of increasing concentrations of fluoride in the absence (○, ●) or presence 100 μM forskolin (△, ▲) at two different concentrations of $MgCl_2$, (●, ▲) the activity obtained in the presence of 5 mM total $MgCl_2$, (○, △) the activities obtained in the presence of 10 mM $MgCl_2$. Adenylyl cyclase activities are expressed in nmol cyclic AMP formed/mg protein in 10 min

Table 2. *Kinetic parameters of liver membrane adenylyl cyclase in the absence and presence of forskolin*
Adenylyl cyclase activity was assayed at varying concentration of ATP in the presence of either 5 mM $MgCl_2$ or 2 mM $MnCl_2$ and apparent K_m values for MnATP and MgATP were derived from Lineweaver-Burk plots. Values represent the mean ± 1/2 range of the parameters assessed in two separate experiments such as those described above. Conditions of incubation were such as to yield linearity of cyclic AMP accumulation throughout the assay times used (10 min) at all concentrations of substrate tested

Substrate	Addition to assays	Apparent K_m	V_{max}
		mM	pmol min^{-1} mg $protein^{-1}$
MgATP	none	0.20 ± 0.02	0.18 ± 0.05
	forskolin (40 μM)	0.22 ± 0.03	2.06 ± 0.08
MnATP	none	0.10 ± 0.02	0.16 ± 0.04
	forskolin (40 μM)	0.13 ± 0.01	2.25 ± 0.50

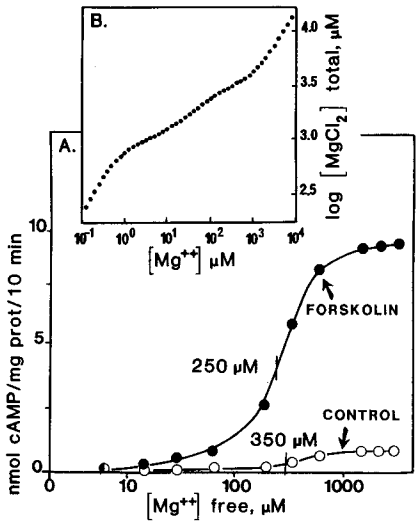

Fig. 4. *Effect of varying the concentration of free Mg on the adenylyl cyclase activity in liver membranes in the absence and presence of 100 μM forskolin.* Liver membranes (20 μg/assay) were incubated in the presence of 2.0 mM [α-^{32}P]ATP (2×10^6 counts/min for each assay), 1.0 mM EDTA, 1.0 mM cyclic [^{3}H]AMP (approx. 10000 counts/min), the ATP-regenerating system described under Materials and Methods, 50 mM Tris/HCl, pH 7.6, and when present, 100 μM forskolin and $MgCl_2$ to give the final concentrations of Mg^{2+} indicated on the *x* axis of the figure. Incubation were for 10 min at 30 °C. The reactions were stopped and the cyclic [^{32}P]AMP formed was determined as described under Materials and Methods. (A) Activities obtained in the absence (○) and presence (●) of forskolin. (B) Standard curve that relates concentration of Mg^{2+} to concentration of total $MgCl_2$ added to media containing 2.0 mM ATP, 1 mM EDTA and 24 mM creatine phosphate at pH 7.6. For further details and methods used to calculate free Mg concentrations, see Materials and Methods

both MgATP and MnATP in Table 2, forskolin did not affect the apparent K_m for either MgATP or MnATP. The stimulatory effect of the diterpene consisted simply of a change in the V_{max} of the system as seen under the assay conditions used (3 mM Mg^{2+} and 2 mM Mn^{2+} in excess of ATP plus EDTA).

The divalent cation requirement for activation of adenylyl cyclases has been shown to be altered by guanine nucleotides, NaF and hormonal stimulation [16–18]. We therefore explored whether forskolin had a similar effect by determining its possible effect on the concentration of added Mg^{2+} (or Mn^{2+}) required to obtain 50 % of maximum activities. Since under the conditions of assay employed the concentration dependence of the system on total added divalent cation is 'steep' and the resultant of the combined formation of MATP and occupancy of allosteric site(s) for M on the enzyme system [16–22], we designed the experiments on the basis of free M^{2+} added instead of total MCl_2 added, i.e. we took quantitatively into consideration the equilibrium dissociation constants of MATP, MEDTA and M creatine phosphate at pH 7.6 (for details see Materials and Methods). As illustrated in Fig. 4 for Mg, it was possible to obtain relatively accurate estimates for the requirement of the system for Mg^{2+} in the absence and presence of forskolin and it was found to be essentially unaltered by the

diterpene, in spite of marked changes in the V_{max}. Since the concentrations of Mg^{2+} giving half-maximal stimulation of activity were well above those needed to obtain the MgATP complex that serves as substrate for the cyclizing reaction, these concentrations (350 μM in the absence and 250 μM in the presence of forskolin) correspond to the apparent K_a values of the allosteric regulatory site or sites of the system for Mg ions. Similar results were obtained when Mn^{2+} was used unstead of Mg^{2+} (not shown).

From the above we concluded that the effect(s) of forskolin do not include modification of either the apparent K_m values for MATP, the substrate, or of the apparent K_a values for M^{2+} at allosteric regulatory site(s).

Effect of proteolytic treatment of liver membranes adenylyl cyclase and its responsiveness to forskolin

Liver membrane adenylyl cyclase activity, like that of other membrane systems, is enhanced upon mild proteolytic treatment in the presence of MgATP (for review see [23]). In view of the additivity and even synergism observed between the effect of forskolin and the various stimulators tested (Table 1), we examined whether forskolin would also stimulate a proteolytically activated adenylyl cyclase. Contrary to findings with guanine nucleotides, fluoride ions and hormone, all of which enhanced forskolin-stimulated activity, proteolytic treatment had no stimulatory action after forskolin stimulation. Rather, as appeared from initial experiments, proteolytic treatment with enzymes such as α chymotrypsin, trypsin and papain under conditions that led to stimulation of basal activity, caused a slight but significant decrease in activities stimulated by saturating concentrations of forskolin. We explored this phenomenon further by assessing the effect of varying concentrations of protease (illustrated for α chymotrypsin on Fig. 5) on basal activity and activities obtained at both saturating and subsaturating concentrations of forskolin. This led to a dissociation of the stimulatory effect of protease from its inhibitory effects. Thus, while at saturating concentrations of forskolin a monotonic concentration-dependent inhibition by protease treatment was seen, we noted that in the absence of forskolin or at low concentrations of the diterpene (10 μM in the example shown in Fig. 5), the protease stimulated activity at low concentrations and inhibited activity at higher concentrations, leading to the well-established biphasic concentration effect curves seen upon protease treatment of adenylyl cyclase systems (cf. [24, 25]). The experiment in Fig. 5 indicates that concentrations of α chymotrypsin, giving apparent 'maximal' stimulation of basal activity, are already inhibitory as seen from the decrease in activity of the fully forskolin-stimulated system. Thus, the so-called 'protease-stimulated' activities are the result of a balance between the stimulating and inhibitory effects of the proteolytic enzymes. The inhibitory effects of protease treatment are not observed when tested for by assaying Guo*PP*[NH]*P* or NaF-stimulated activities (Table 3).

*Effect(s) of apparent 'uncoupling' of regulatory component from the catalytic unit by high Mn ion concentration on forskolin, Guo*PP*[NH]*P *and NaF stimulation of liver membrane adenylyl cyclase*

Limbird et al. [26] reported that concentrations in excess of 20 mM Mn ions 'uncouple' the guanine-nucleotide-binding regulatory component from the catalytic unit of adenylyl cyclase without interfering with the regulatory effects of the

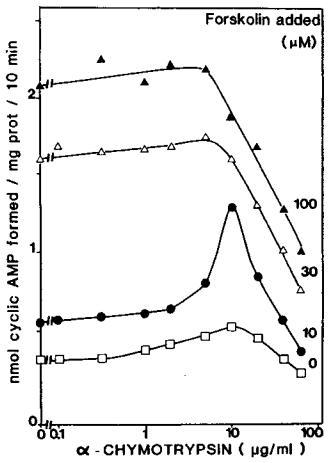

Fig. 5. *Effect of α chymotrypsin on forskolin stimulation of adenylyl cyclase activities.* Rat liver plasma membranes were incubated in the presence of increasing amounts of α chymotrypsin in the presence of MgATP and in the absence (□) or presence of 10 μM (●), 30 μM (△) and 100 μM (▲) forskolin for 10 min at 30 °C. Adenylyl cyclase activities are expressed in nmol cyclic AMP formed/mg protein in 10 min

Table 3. *Effect of proteolytic treatment of liver membranes on adenylyl cyclase activity assayed in the absence and presence of forskolin, Guo*PP*[NH]*P *and NaF*

Liver membranes (1.1 mg) were treated for 5 min at 30 °C in a final volume of 0.5 ml with 1 μg/ml or 8 μg/ml α chymotrypsin. At the end of the treatment period, soybean trypsin inhibitor was added to give a final concentration of 0.4 mg/ml and the incubation was contained for 2 min at 30 °C to stop α chymotryptic action. Next the mixtures were diluted twice with 50 mM Tris pH 7.6, placed on ice and aliquots (20 μl) were assayed for adenylyl cyclase activity as described under Material and Methods in the presence of the addition listed below. Values are mean ± 1/2 range of adenylyl cyclase activities determined in duplicate. When present, forskolin was 100 μM, Guo*PP*[NH]*P* was 10 μM and NaF was 10 mM

Addition during assay	α-Chymotrypsin addition during treatment		
	none	1 μg/ml	8 μg/ml
	nmol cyclic AMP formed/mg in 10 min		
None	0.18 ± 0.02	0.26 ± 0.02	0.26 ± 0.03
Forskolin	2.92 ± 0.03	2.68 ± 0.03	1.43 ± 0.05
Guo*PP*[NH]*P*	2.04 ± 0.04	2.24 ± 0.08	2.45 ± 0.03
Guo*PP*[NH]*P* + forskolin	4.44 ± 0.09	4.65 ± 0.10	3.79 ± 0.12
NaF	1.17 ± 0.05	1.53 ± 0.14	1.72 ± 0.02
NaF + forskolin	3.20 ± 0.18	2.54 ± 0.02	2.38 ± 0.02

nucleotide-binding protein on agonist – receptor interactions. We carried out experiments to test whether such 'uncoupled' systems are responsive to forskolin. To this end we incubated liver membranes in two stages: a first stage or treatment, in which we promoted uncoupling, and a second stage or incubation proper where we assayed for adenylyl cyclase activity in the absence and presence of guanine nucleotide Guo*PP*[NH]*P* forskolin and, in some cases, NaF. In the course of these experiments we varied the concentration of divalent cations during both treatment and incubation in order to gain a better insight into reasons for the changes that we observed. Table 4 presents the results of three representative experiments that summarize our findings.

Table 4. *Effect of forskolin on 'Mn^{2+}-uncoupled' liver membrane adenylyl cyclase activity*

Membranes were treated at 20 °C in a final volume of 40 µl with 30 mM $MnCl_2$ or 5 mM $MgCl_2$ in the presence of 0.6 mM ATP, 1.2 mM EDTA, 1.2 mM cyclic [^{3}H]AMP, the ATP-regenerating system described under Materials and Methods, 50 mM Tris/HCl pH 7.6 and, when present, 120 µM forskolin or 12 mM NaF. After 10–15 min, 20 µl medium were added to complete adenylyl cyclase assay conditions and when required to added 10 µM Guo*PP*[NH]*P*, 10 mM NaF and 100 µM forskolin. The reactions were stopped and cyclic [^{32}P]AMP formed was determined as described under Materials and Methods. When present, forskolin was 100 µM, NaF was 10 mM. When added Guo*PP*[NH]*P* and NaF were 10 µM and 10 mM, respectively, and forskolin was an additional 100 µM. Values are means ± SD of triplicate determinations and are given as nmol cyclic AMP/mg protein in the period of the assay time. In experiment I treatment was for 10 min, assays were for 10 min and liver membranes were 20 µg/assay. In experiment II treatment was for 15 min, assays were for 15 min and liver membranes were 18 µg/assay. In experiment III treatment was for 10 min, assays were for 15 min, and liver membranes were 15 µg/assay

Additions to		Adenylyl cyclase activity
treatment	assay	
		nmol cyclic AMP/mg
Experiment I		
A. Treatment and assay at 30 mM $MnCl_2$		
None	none	0.21 ± 0.02
None	Guo*PP*[NH]*P*	0.28 ± 0.02
None	forskolin	1.35 ± 0.10
None	NaF	0.40 ± 0.03
B. Treatment and assay at 5 mM $MgCl_2$		
None	none	0.20 ± 0.02
None	Guo*PP*[NH]*P*	1.90 ± 0.10
None	forskolin	2.90 ± 0.25
None	NaF	1.40 ± 0.22
Experiment II		
Treatment at 5 mM $MgCl_2$, assay at 30 mM $MnCl_2$		
None	none	0.74 ± 0.05
None	Guo*PP*[NH]*P*	0.80 ± 0.03
None	forskolin	8.86 ± 0.26
None	forskolin + Guo*PP*[NH]*P*	6.58 ± 0.97
Forskolin	forskolin	12.24 ± 0.42
Forskolin	forskolin + Guo*PP*[NH]*P*	10.35 ± 1.41
Experiment III		
Treatment and assay at 30 mM $MnCl_2$		
None	none	0.61 ± 0.09
None	Guo*PP*[NH]*P*	0.80 ± 0.05
None	NaF	1.00 ± 0.05
None	forskolin	2.22 ± 0.20
NaF	NaF	2.25 ± 0.19
Forskolin	forskolin	3.42 ± 0.11

In part A of experiment I and in experiment II of Table 4 we confirmed the report from Lefkowitz and collaborators [26] that assay of membranes in the presence of 30 mM $MnCl_2$ leads to a loss of the effect of Guo*PP*[NH]*P*. In part A of experiment I and in experiment III we pretreated membranes with 30 mM $MnCl_2$ and assayed them with 30 mM $MnCl_2$. In experiment II we pretreated the membranes with 5 mM $MgCl_2$ and then assayed activity in the presence of excess (30 mM) $MnCl_2$. In part B of experiment I both stages of incubation were carried out at 5 mM $MgCl_2$. This constitutes a control in which we tested the degree of stimulation by Guo*PP*[NH]*P* we would have obtained if no inhibition of action had occurred. Guo*PP*[NH]*P*, when present, was added at $t = 0$ of the second stage of incubation. It is clear from the data shown in Table 4 that $MnCl_2$ in the assay totally abolishes the effect of the guanine nucleotide regardless of whether the membranes are pretreated with $MnCl_2$. Upon testing the effect of forskolin addition under conditions where no Guo*PP*[NH]*P* effect is elicited, we found that the action of the diterpene was not abolished concomitantly with that of the nucleotide (part A of experiment I, and experiments II and III). The effect of forskolin did not appear to be associated with a 'recoupling' of the system, since a combination of forskolin and Guo*PP*[NH]*P*, which in control incubations elicits additive effects, led to more activity than that obtained with forskolin alone. We noticed, however, that a pretreatment of membranes in the presence of $MnCl_2$ in the absence of forskolin resulted in a significantly lower forskolin-stimulated activity than when pretreatment was carried out in the presence of forskolin. This increased forskolin-stimulated activity, obtained upon addition of forskolin during the pretreatment, appears to be due to a protecting effect of the diterpene, because (a) a similar result can be obtained with NaF (experiment III) and (b) forskolin was found in other experiments, not reported here, to stabilize catalytic activity to heat denaturation, a phenomenon that is enhanced in the experiments of Table 4 due to the presence of 30 mM $MnCl_2$.

DISCUSSION

The studies presented above characterize the properties of the forskolin-stimulated liver membrane adenylyl cyclase. As found for other systems the diterpene is a potent stimulator of liver membrane adenylyl cyclase, eliciting activities that were higher than those of obtained by either NaF or glucagon. Combinations of forskolin with NaF or glucagon yielded additive effects under the conditions of assay used. The kinetic mechanism, by which forskolin affects adenylyl cyclase, differs remarkably from that used by guanine nucleotides of NaF. Thus, while the nucleotides and fluoride ions enhance activity with concomitant decrease in apparent K_m for Mg ions [16, 17], forskolin only affected the V_{max} of the system leaving unaltered the apparent K_m for both divalent cation and substrate (Fig. 4, Table 2). Forskolin also did not affect significantly the potency (apparent K_a) of the action of the GTP analog Guo*PP*[NH]*P* NaF or hormone (Fig. 2 and 3). The question may be raised on the basis of these results whether forskolin acts on the same adenylyl cyclase as that affected by NaF and hormone. However, the findings illustrated in Fig. 5 and Table 3, showing that proteolytic enhancement of either basal, Guo*PP*[NH]*P*-stimulated or NaF-stimulated activities was not additive with that of forskolin, argue that a single system is regulated by all stimulators.

The findings that forskolin stimulated adenylyl cyclase activity after 'uncoupling' of the stimulatory effects of guanine nucleotides, an effect that did not appear to be due to a 'recoupling' of the system (Table 4), clearly indicate that the mechanism(s) involved in the action of forskolin differs fundamentally from that involved in guanine nucleotide action. The total lack of effect of forskolin on the requirement of the system for Mg or Mn (illustrated for Mg in Fig. 4) a parameter that is affected by NaF, indicates that the diterpene also acts differently from the way NaF acts.

The different in the mode of action of forskolin compared to that of nucleotides and NaF is of importance, for it indicates the potential existence of as yet undiscovered additional ways of regulating cyclic AMP levels in cells. It raises the intriguing question of what 'endogenous forskolin' may be. The actual molecular mechanism by which forskolin acts as well as the location of the site to which it attaches to exert its action is a matter for discussion and future experimentation.

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L. Birnbaumer, Department of Cell Biology, Baylor College of Medicine, Houston, Texas, USA 77030

D. Stengel, M. Desmier, and J. Hanoune, Unité de Recherches sur les Métabolismes, les Agents Thérapeutiques et l'Appareil Digestif (Unité 99 de l'Institut National de la Santé et de la Recherche Médicale), Hôpital Henri-Mondor, 51 Avenue du Maréchal-de-Lattre-de-Tassigny, F-94010 Créteil, Val-de-Marne, France